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**TLR2 antagonistic antibody and use thereof**

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The present invention is directed to a cross-reactive antibody, which specifically inhibits or blocks the mammalian Toll-like receptor 2 (TLR2)-mediated immune cell activation. The invention is further directed to an isolated nucleic acid or vector coding for the variable regions of the heavy and/or light chain of said antibody. It is further providing a pharmaceutical composition comprising said antibody, nucleic acid or vector and is directed to the use of said composition in the prevention and/or treatment of inflammatory processes or any other process induced by bacterial infection, trauma, or chronic inflammation, or for the prevention and/or treatment of bacteraemia or sepsis.

Host cells recognize specific patterns of microbial components through Toll-like receptors (TLRs) which are crucial in mediating innate immune responses<sup>1,2</sup>. Lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria is a potent agonist for TLR4 whose effects on the host organism have been studied extensively in experimental models of infection and septic shock<sup>3-5</sup>. Over-stimulation of host immune cells by microbial products accompanied by the release of large amounts of inflammatory mediators is recognized as a major cause of septic shock<sup>6-9</sup>.

Indeed, this concept has been validated by using both gene targeted mice lacking the expression of the respective receptors, and by receptor specific inhibition of microbial product induced host cell activation. For example, the non-redundant role of CD14 as an important element of a cellular LPS recognition system has been demonstrated by application of inhibitory anti CD14 antibodies in rabbits<sup>10,11</sup>. The blockade of LPS receptors or extracellular effector proteins as the earliest possible targets of therapeutic strategies was shown to be preventive<sup>12</sup>. Another approach of therapeutic intervention in septic shock has been interference with the functions of proinflammatory cytokines such as TNF $\alpha$  or IL-1 $\beta$ . For instance, competitive inhibition of cytokine binding to its signaling receptors by

application of recombinant extracellular domain (ECD) or naturalizing receptor antagonist proteins have been shown to be protective in LPS induced shock in rats<sup>13</sup>. In addition, antagonistic antibodies targeting cytokines or ECDs of its receptors have been tested<sup>14</sup>. While cytokine blockade for therapeutic intervention in acute infections (sepsis) has been disappointing<sup>15</sup>, its use in treatment of chronic inflammations is promising<sup>16,17</sup>.

Besides Gram-negative, Gram-positive bacteria lacking LPS play an important role in the clinical manifestation of shock<sup>8</sup>. Cell wall components from these bacteria such as peptidoglycan (PGN) and lipoteichoic acid (LTA) are considered the main causative agents of Gram-positive shock<sup>18,19</sup>. PGN is a main component of Gram-positive, but also of other bacterial cell walls, and consists of an alternating  $\beta$  (1,4) linked N-acetylmuramyl and N-acetylglucosaminyl glycan cross linked by small peptides<sup>20</sup>. In contrast, the macroamphiphile LTA, a saccharide chain molecule consisting of repetitive oligosaccharides connected by alcohols such as ribitol and carrying acyl chains through which it is anchored to the bacterial cytoplasmic membrane, is specific for Gram-positive bacteria<sup>21</sup>. For example, LTA has been described to carry the major stimulatory activity of *Bacillus subtilis*<sup>22</sup>. The stimulatory properties of tripalmitoylated proteins whose production is not restricted to Gram-negative or Gram-positive bacteria are mimicked by the synthetic compound N-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2R,S)-propyl)-(R)-cysteinyl-seryl-(lysyl)3-lysine ( $P_3CSK_4$ )<sup>23</sup>.

Most of these bacterial products are known to activate innate immune cell responses by triggering the TLR2 signaling cascade<sup>2</sup>. The TLR2ECD, whose N-terminal portion has been implicated in PGN recognition<sup>24</sup>, contains an array of distinct leucine rich repeat (LRR) motifs<sup>25</sup>. The LRR rich domain is followed by an LRR C-terminal (LRRCT), a trans-membrane, and an intracellular C-terminal Toll – IL-1 receptor – plant disease resistance protein (TIR) domain<sup>25</sup>.

WO 01/36488 generally describes an anti-TLR2 antibody. It is further described to prepare such an antibody by using a TLR2 immunogen, for example a part of the TLR2 molecule, for example a domain (e. g. the extracellular domain) or a shorter peptide or polypeptide sequence e. g. an epitopic sequence. However, it is indicated in WO 01/36488 that it is preferred for the immunogen to be one which mimics the natural protein as far as possible.

WO 01/36488 in particular obtains and discloses a monoclonal antibody having particular properties and characteristics (e. g. binding and inhibition characteristics). This antibody is called „Mab TL2.1“. The hybridoma producing this antibody was deposited under the terms of the Budapest Treaty at the European Collection of Cell Cultures (ECACC) on October 28, 1999 under the Accession No. 99102832. This antibody is the only precise and fully disclosed Example for an antibody presented in WO 01/36488.

The inventors conducted some research work and tested Mab TL2.1 for its characteristics and it turned out that this antibody is not cross-reactive between different mammalian species.

Therefore, it is the objective problem underlying the present invention to provide a cross-reactive antibody, which specifically inhibits or blocks the mammalian Toll-like receptor 2 (TLR2)-mediated immune cell activation by specifically binding to the highly conserved C-terminal portion of the extracellular domains of TLR2. It is further an objective problem of the present invention to provide an antibody which is capable of prevention and/or treatment of bacteraemia or sepsis, or of prevention and/or treatment of inflammatory processes or any other process induced by bacterial infection, trauma, or chronic inflammation. Additionally, a problem underlying the invention is to provide an improved approach of gene therapy, based on an overexpression of the antibody or a functional part thereof in a host organism in order to achieve the above mentioned therapeutic effect.

These objects are solved by the subject-matters of the independent claims. Preferred embodiments are set forth in the dependent claims.

Herein, it is shown that a murine monoclonal antibody (mAb) raised against the murine TLR2 extracellular domain (=ECD) inhibits TLR2 mediated activation of murine and human cells. The protective potential of neutralizing TLR2 with this antibody was demonstrated *in vivo* using two TLR2 dependent shock models. Thus, antibody targeting of the TLR2ECD is a valuable strategy to prevent TLR2 driven septic shock.

In this invention, for the first time a cross-reactive antibody is described, which is capable of inhibiting or blocking the mammalian Toll-like receptor 2 (TLR2)-mediated immune cell activation by specifically binding to the C-terminal portion of the extracellular domains of at least human and murine TLR2. The cross-reactivity of the antibody of the invention in particular was shown by specificity for the human and murine TLR2 sequences. Thus, the antibody of the present invention for the first time is capable of specifically binding to the highly conserved TLR2 sequence shared among most mammals, in particular among mice and human beings.

It is noted that according to experimental data obtained by the inventors, the presently known antibodies as disclosed in WO 01/36488 (TL2.1) interact with human TLR2 and an unspecific antigen from murine cells in FACS analysis and immunocytochemical staining analysis whereas the antibody of the present invention (e.g. T2.5) specifically recognizes both murine and human TLR2, therefore being cross-reactive. The respective experimental data are provided in the Figures and the Examples.

The present invention in particular is directed to the following aspects and embodiments:

According to a first aspect, the present invention is directed to a cross-reactive antibody, which specifically inhibits or blocks the mammalian Toll-like receptor 2 (TLR2)-mediated immune cell activation by specifically binding to the C-terminal portion of the extracellular domains of at least human and murine TLR2.

As mentioned above, the antibody of the present invention is defined as being cross-reactive as an essential feature. The term "cross-reactive" as used herein refers to the ability of an individual antibody to react with an antigenic determinant within the TLR2 orthologue products of two different species (here: human and murine). Cross reactivity in the meaning of the present invention arises because one specific antigen has an epitope which is structurally similar to the analogous epitope within the analogous protein of two species. As mentioned above, an example of cross-reactivity in the meaning of the present invention is the capability of an anti-TLR2 antibody to specifically bind to the C-terminal portion of the extracellular domains of at least human and murine TLR2 which is preferably encompassing

a region from amino acids T221 to R587 (for the corresponding sequence information it is referred to Genebank accession number HSU 88878) or a subregion thereof.

The term extracellular domain is used herein as commonly defined in the corresponding field of science, i.e. as usually defined in the field of type I transmembrane receptors. Those are proteins that span the thickness of the plasma membrane of the cell, with one end of the receptor outside (*extracellular domain*) and one inside (*intracellular domain*) the cell. Both are connected by the transmembrane domain.

The antibody of the present invention is, according to an embodiment, a polyclonal antibody, a monoclonal antibody, a humanized antibody, a chimeric antibody, or a synthetic antibody.

The term “antibody”, is used herein for intact antibodies as well as antibody fragments, which have a certain ability to selectively bind to an epitop. Such fragments include, without limitations and as an example, Fab, F(ab')<sub>2</sub> und Fv antibody fragment. The term “epitop” means any antigen determinant of an antigen, to which the paratop of an antibody can bind. Epitop determinants usually consist of chemically active surface groups of molecules (e.g. amino acid or sugar residues) and usually display a three-dimensional structure as well as specific physical properties.

The antibodies according to the invention can be produced according to any known procedure. For example, the pure complete TLR2 ECD or a part of it can be produced and used as immunogen, to immunize an animal and to produce specific antibodies.

The production of polyclonal antibodies is commonly known. Detailed protocols can be found for example in Green et al, Production of Polyclonal Antisera, in *Immunochemical Protocols* (Manson, editor), pages 1 - 5 (Humana Press 1992) und Coligan et al, Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters, in *Current Protocols In Immunology*, section 2.4.1 (1992). In addition, the expert is familiar with several techniques regarding the purification and concentration of polyclonal antibodies, as well as of monoclonal antibodies (Coligan et al, Unit 9, *Current Protocols in Immunology*, Wiley Interscience, 1994).

The production of monoclonal antibodies is as well commonly known. Examples include the hybridoma method (Kohler and Milstein, 1975, *Nature*, 256:495-497, Coligan et al., section 2.5.1 - 2.6.7; and Harlow et al., *Antibodies: A Laboratory Manual*, page 726 (Cold Spring Harbor Pub. 1988).), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96).

In brief, monoclonal antibodies can be attained by injecting a mixture which contains the protein in question into mice. The antibody production in the mice is checked via a serum probe. In the case of a sufficient antibody titer, the mouse is sacrificed and the spleen is removed to isolate B-cells. The B cells are fused with myeloma cells resulting in hybridomas. The hybridomas are cloned and the clones are analyzed. Positive clones which contain a monoclonal antibody against the protein are selected and the antibodies are isolated from the hybridoma cultures. There are many well established techniques to isolate and purify monoclonal antibodies. Such techniques include affinity chromatography with protein A sepharose, size-exclusion chromatography and ion exchange chromatography. Also see for example, Coligan et al., section 2.7.1 – 2.7.12 and section „Immunglobulin G (IgG)“, in *Methods In Molecular Biology*, volume 10, pages 79 – 104 (Humana Press 1992).

According to a preferred embodiment, the antibody of the invention specifically binds through its variable regions of the heavy- and light chain, which regions carry the amino acid sequence as depicted in SEQ ID NO:1 and/or 2, or a variant thereof. The term “variant” as used in this connection is explained in detail below (regarding the aspect directed to the nucleic acid of the invention).

Methods of making such antibody fragments, and synthetic and derivatised antibodies are well known in the art. Also included in the scope of the term „antibody“ as used herein (in addition to the ones indicated above) are antibody fragments containing the complementarity-determining regions (CDRs) or hypervariable regions of the antibodies. These may be defined as the region comprising the amino acid sequences on the light and heavy chains of an antibody which form the three-dimensional loop structure that contributes to the formation of

the antigen binding site. CDRs may be used to generate CDR-grafted antibodies. The term "CDR grafted" defines an antibody having an amino acid sequence in which at least parts of one or more sequences in the light and/or variable domains have been replaced by analogous parts of CDR sequences from an antibody having a different binding specificity for a given antigen.

One of skill in the art can readily produce such CDR grafted antibodies using methods well known in the art (see Borrebaeck, Antibody Engineering: A Practical Guide, W. H. Freeman and Company, New York, 1992).

A chimeric antibody of the present invention may be prepared by combining the variable region of an anti TLR2 antibody (or a part thereof) of one species according to the present invention with the constant regions of an antibody derived from a different species. A chimeric antibody may be constructed, for example, according to methods described by Shaw et al., in J. Immun. 1987, 138: 4534 and Sun et al., in PNAS USA, 1987, 84: 214-218.

Monoclonal antibodies and their fragments and derivatives are preferred antibodies according to the present invention.

A further aspect of the invention is thus a hybridoma or cell-line producing an antibody of the invention as defined above.

According to a further embodiment, the antibody of the invention is linked to a pharmaceutical agent and/or to a detectable agent.

In a second aspect, the present invention is directed to an isolated nucleic acid coding for the variable regions of the heavy and/or light chain of the antibody as defined above.

In particular, the invention is directed to an isolated nucleic acid which comprises the sequence of SEQ ID NO: 1 and/or 2 or variants thereof, wherein the variants are each defined as having one or more substitutions, insertions and/or deletions as compared to the sequence of SEQ ID NO: 1 and/or 2, provided that said variants hybridize under moderately stringent

conditions to a nucleic acid which comprises the sequence of SEQ ID NO: 1 and/or 2, and further provided that said variants code for an amino acid having activity as a variable region of an antibody specifically binding to the C-terminal portion of the extracellular domains of at least human and murine TLR2 or provided that said variants comprise nucleic acid changes due to the degeneracy of the genetic code, which code for the same or a functionally equivalent amino acid as the nucleic acid sequence of SEQ ID NO: 1 and/or 2.

More preferably, the isolated nucleic acid of the invention comprises at least the sequence of nucleic acids No. 172 – 201, 244 – 294 and/or 385 – 417 of SEQ ID NO: 1, or of nucleic acids No. 130 – 174, 220 – 240 and/or 337 – 363 of SEQ ID NO: 2, or parts thereof. The above mentioned sequences comprise complementarity determining regions (CDR's) 1, 2 and 3, respectively.

Regarding SEQ ID NO: 1, more precisely, nucleic acids No. 172-195, 247-270 and 385-417 or parts thereof are preferred (CDR 1 – 3), which were identified by application of the IMGT database. Alternatively, nucleic acids 187-201 and 244-294 (CDR 1 and 2) are preferred, identified by the V-BASE database.

Regarding SEQ ID NO: 2, more precisely, nucleic acids No. 139-168, 220-228 and 337-363 or parts thereof are preferred (CDR 1 – 3), which were identified by application of the IMGT database. Alternatively, nucleic acids 130-174 and 220-240 (CDR 1 and 2) are preferred, identified by the V-BASE database.

A chart comprising SEQ ID NO: 1 and its complementary sequence as well as the encoded amino acids are depicted below. SEQ ID NO: 1 is showing the nucleic acid and amino acid sequence of the heavy chain's variable region of the antibody of the invention.

SEQ ID NO:1            1 to 501

10	20	30	40	50
ATGTCCTCTC	CACAGTCCCT	GAAGACACTG	ATTCTAACCA	TGGGATGGAG
TACAGGAGAG	GTGTCAGGGA	CTTCTGTGAC	TAAGATTGGT	ACCCTACCTC
MetSerSer	ProGlnSerLeu	LysThrLeu	IleLeuThr	MetGlyTrpSer>
60	70	80	90	100
CTGGATCTTT	CTCTTCCTCC	TGTCAGGAAC	TGCAGGTGTC	CACTCCCAGG
GACCTAGAAA	GAGAAGGAGG	ACAGTCCTTG	ACGTCCACAG	GTGAGGGTCC



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TrpIlePhe LeuPheLeu LeuSerGlyThr AlaGlyVal HisSerGln>

      110      120      130      140      150
TTCAGCTGCA GCAGTCTGGA CCTGAGCTGG TGAACCTGG GGCCTCAGTG
AAGTCGACGT CGTCAGACCT GGACTCGACC ACTTGGGACC CCGCAGTCAC
ValGlnLeuGln GlnSerGly ProGluLeu ValAsnProGly AlaSerVal>

      160      170      180      190      200
AAGTTGTCCT GCAAGGCTTC TGGCTTCACC TTCACAACCT ACGGTATAAA
TTCAACAGGA CGTTCCGAAG ACCGAAGTGG AAGTGTGGA TGCCATATTT
LysLeuSer CysLysAlaSer GlyPheThr PheThrThr TyrGlyIleAsn>

      210      220      230      240      250
CTGGGTGAAG CAGGGGCCTG GACAGGGACT TGAGTGGATT GGATGGATTT
GACCCACTTC GTCCCCGGAC CTGTCCCTGA ACTCACCTAA CCTACCTAAA
TrpValLys GlnGlyPro GlyGlnGlyLeu GluTrpIle GlyTrpIle>

      260      270      280      290      300
ATCCTAGAGA TGGTAGTACT AACTTCAATG AGAATTTCAA GGACAAGGCC
TAGGATCTCT ACCATCATGA TTGAAGTTAC TCTTAAAGTT CCGTTTCCGG
TyrProArgAsp GlySerThr AsnPheAsn GluAsnPheLys AspLysAla>

      310      320      330      340      350
GCATTGACTG TAGACACATC CTCCAGCACA GCGTACATGG AACTCCACAG
CGTAACTGAC ATCTGTGTAG GAGGTCGTGT CGCATGTACC TTGAGGTGTC
AlaLeuThr ValAspThrSer SerSerThr AlaTyrMet GluLeuHisSer>

      360      370      380      390      400
CCTGACATCT GAAGACTCTG CGGTCTATTT CTGTGCAAGA CTGACTGGTG
GGACTGTAGA CTTCTGAGAC GCCAGATAAA GACACGTTCT GACTGACCAC
LeuThrSer GluAspSer AlaValTyrPhe CysAlaArg LeuThrGly>

      410      420      430      440      450
GGACATTCTT TGACTATTGG GGCCAGGGCA CCACTCTCAC AGTCTCCTCA
CCTGTAAGGA ACTGATAACC CCGGTCCCGT GGTGAGAGTG TCAGAGGAGT
GlyThrPheLeu AspTyrTrp GlyGlnGly ThrThrLeuThr ValSerSer>

      460      470      480      490      500
GCCAAAACGA CACCCCCATC TGTCTATCCA CTGGCCCCTG GATCTGCTGC
CGGTTTTGCT GTGGGGGTAG ACAGATAGGT GACCGGGGAC CTAGACGACG
AlaLysThr ThrProProSer ValTyrPro LeuAlaPro GlySerAlaAla>

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It is noted that the underlined regions are CDR1 and CDR2 sequentially defined by usage of V-BASE database, both nucleic acid and amino acid sequences are underlined accordingly. Italic/bold regions are CDR 1 to 3 defined by application of IMGT database, both nucleic acid and amino acid sequences are marked accordingly (applies also to SEQ ID NO: 2).

Further, a chart comprising SEQ ID NO: 2 and its complementary sequence as well as the encoded amino acids are depicted below. SEQ ID NO: 2 is showing the nucleic acid and amino acid sequence of the light chain's variable region of the antibody of the invention.

SEQ ID NO: 2 1 to 443

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      10      20      30      40      50
ATGGAGTCAG ACACACTCCT GCTATGGGTG CTGCTGCTCT GGGTTCACAGG
TACCTCAGTC TGTGTGAGGA CGATACCCAC GACGACGAGA CCCAAGGTCC
MetGluSer AspThrLeuLeu LeuTrpVal LeuLeuLeu TrpValProGly>

      60      70      80      90     100
CTCCACTGGT GACATTGTGC TCACCCAATC TCCAGCTTCT TTGGCTGTGT
GAGGTGACCA CTGTAACACG AGTGGGTTAG AGGTCAAGA AACCGACACA
SerThrGly AspIleVal LeuThrGlnSer ProAlaSer LeuAlaVal>

     110     120     130     140     150
CTCTAGGGCA GAGAGCCACC ATCTCCTGCA GAGCCAGTGA AAGTGTGAA
GAGATCCCGT CTCTCGGTGG TAGAGGACGT CTCGGTCACT TTCACAACCT
SerLeuGlyGln ArgAlaThr IleSerCys ArgAlaSerGlu SerValGlu>

     160     170     180     190     200
TATTATGGCA CAAGTTTAAT GCAGTGGTAC CAACAGAAAC CAGGACAGCC
ATAATACCGT GTTCAAATTA CGTCACCATG GTTGTCTTTG GTCCTGTCCG
TyrTyrGly ThrSerLeuMet GlnTrpTyr GlnGlnLys ProGlyGlnPro>

     210     220     230     240     250
ACCCAAACTC CTCATCTTTG GTGCATCCAA CGTAGAATCT GGGGTCCCTG
TGGGTTTGAG GAGTAGAAAC CACGTAGGTT GCATCTTAGA CCCCAGGGAC
ProLysLeu LeuIlePhe GlyAlaSerAsn ValGluSer GlyValPro>

     260     270     280     290     300
TCAGGTTTCAG TGGCAGTGGG TCTGGGACAG ACTTCAGCCT CAACATCCAT
AGTCCAAGTC ACCGTCACCC AGACCCTGTC TGAAGTCGGA GTTGTAGGTA
ValArgPheSer GlySerGly SerGlyThr AspPheSerLeu AsnIleHis>

     310     320     330     340     350
CCTGTGGAGG AGGATGATAT TGTAATGTAT TTCTGTCAGC AAAGTAGGAA
GGACACCTCC TCCTACTATA ACATTACATA AAGACAGTCG TTTCATCCTT
ProValGlu GluAspAspIle ValMetTyr PheCysGln GlnSerArgLys>

     360     370     380     390     400
ACTTCCGTGG ACGTTCGGTG GAGGCACCAA GCTGGAAATC AAACGGGCTG
TGAAGGCACC TGCAAGCCAC CTCCGTGGTT CGACCTTTAG TTTGCCCCGAC
LeuProTrp ThrPheGly GlyGlyThrLys LeuGluIle LysArgAla>

     410     420     430     440
ATGCTGCACC AACTGTATCC ATCTTCCCAC CATCCAGTGA GCA
TACGACGTGG TTGACATAGG TAGAAGGGTG GTAGGTCCT CGT
AspAlaAlaPro ThrValSer IlePhePro ProSerSerGlu Xxx>

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Furthermore, the whole sequence of the variable region of the heavy chain (including a 5' non coding region) reads as follows:

SEQ ID NO: 3:

CATGGACTGAAGGAGTAGAAAAGACAACCTATGGCCAATGTCCTCTCCACAGTCCC  
TGAAGACACTGATTCTAACCATGGGATGGAGCTGGATCTTTCTCTTCCTCCTGTCA  
GGAAGTGCAGGTGTCCACTCCCAGGTTTCAGCTGCAGCAGTCTGGACCTGAGCTGG  
TGAACCTTGGGGCGTCAGTGAAGTTGTCCTGCAAGGCTTCTGGCTTCACCTTCAC  
AACCTACGGTATAAACTGGGTGAAGCAGGGGCCTGGACAGGGACTTGAGTGGAT  
TGGATGGATTTATCCTAGAGATGGTAGTACTAACTTCAATGAGAATTTCAAGGAC  
AAGGCCGCATTGACTGTAGACACATCCTCCAGCACAGCGTACATGGAAGTCCACA  
GCCTGACATCTGAAGACTCTGCGGTCTATTTCTGTGCAAGACTGACTGGTGGGAC  
ATTCCTTGACTATTGGGGCCAGGGCACCACTCTCACAGTCTCCTCAGCCAAAACG  
ACACCCCCATCTGTCTATCCACTGGCCCCCTGGATCTGCTGCC

The whole sequence of the variable region of the light chain (including a 5' non coding region) reads as follows:

SEQ ID NO:4:

CATGGACTGAAGGAGTAGAAAATCCTCTCATCTAGCTCTCAGAGATGGAGTCAGA  
CACACTCCTGCTATGGGTGCTGCTGCTCTGGGTTCCAGGCTCCACTGGTGACATTG  
TGCTCACCCAATCTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGAGAGCCACCATC  
TCCTGCAGAGCCAGTGAAAGTGTTGAATATTATGGCACAAGTTTAATGCAGTGGT  
ACCAACAGAAACCAGGACAGCCACCCAACTCCTCATCTTTGGTGCATCCAACGT  
AGAATCTGGGGTCCCTGTCAGGTTTCAGTGGCAGTGGGTCTGGGACAGACTTCAGC  
CTCAACATCCATCCTGTGGAGGAGGATGATATTGTAATGTATTTCTGTCAGCAAA  
GTAGGAAACTTCCGTGGACGTTTCGGTGGAGGCACCAAGCTGGAAATCAAACGGG  
CTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCA

The nucleic acid variants according to the invention also comprise nucleic acid fragments which contain more than 10, preferably more than 15, more than 20, more than 25 or more than 30 and up to 50 nucleotides. Most preferably, the fragments comprise the CDR regions as indicated above.

According to the state of the art an expert can test which derivatives and possible variations derived from these revealed nucleic acid sequences according to the invention are, are partially or are not appropriate for specific applications.

Amplification and detection methods are according to the state of the art. The methods are described in detail in protocol books which are known to the expert. Such books are for

example Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, and all subsequent editions. PCR-methods are described for example in Newton, PCR, BIOS Scientific Publishers Limited, 1994 and all subsequent editions.

As defined above, “variants” are according to the invention especially such nucleic acids, which contain one or more substitutions, insertions and or deletions when compared to the nucleic acids of SEQ ID NO: 1 and 2. These lack preferably one, but also 2, 3, 4, or more nucleotides 5’ or 3’ or within the nucleic acid sequence, or these nucleotides are replaced by others.

The nucleic acid sequences of the present invention comprise also such nucleic acids which contain sequences in essence equivalent to the nucleic acids described in SEQ ID NO: 1 and 2. According to the invention nucleic acids can show for example at least about 80%, more typically at least about 90% or 95% sequence identity to the nucleic acids described in SEQ ID NO: 1 and 2.

It is noted that the above considerations also apply to SEQ ID NO: 3 and 4.

The term “nucleic acid sequence” means a heteropolymer of nucleotides or the sequence of these nucleotides. The term “nucleic acid”, as herein used, comprises RNA as well as DNA including cDNA, genomic DNA and synthetic (e.g. chemically synthesized) DNA and other polymer linked bases such as PNA (peptide nucleic acids).

The invention comprises – as mentioned above – also such variants which hybridize to the nucleic acids according to the invention under moderately stringent conditions.

Stringent hybridization and wash conditions are in general the reaction conditions for the formation of duplexes between oligonucleotides and the desired target molecules (perfect hybrids) or that only the desired target can be detected. Stringent washing conditions mean, e.g., 0.2 x SSC (0.03 M NaCl, 0.003 M sodium citrate, pH 7)/0.1% SDS at 65°C. For shorter fragments, e.g. oligonucleotides up to 30 nucleotides, the hybridization temperature is below 65°C, for example at 50°C, preferably above 55°C, but below 65°C. Stringent hybridization

temperatures are dependent on the size or length, respectively of the nucleic acid and their nucleic acid composition and will be experimentally determined by the skilled artisan. Moderately stringent hybridization temperatures are for example 42°C and washing conditions with 0.2 x SSC/0.1% SDS at 42°C.

Also contemplated herein are amino acid sequences of the above identified kind, which also encompass all sequences differing from the herein disclosed sequences by amino acid insertions, deletions, and substitutions.

Preferably, amino acid "substitutions" are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, i.e., conservative amino acid replacements. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

"Insertions" or "deletions" are typically in the range of about 1 to 5 amino acids. The variation allowed may be experimentally determined by systematically making insertions, deletions, or substitutions of amino acids in a polypeptide molecule using recombinant DNA techniques and assaying the resulting recombinant variants for activity. This does not require more than routine experiments for the skilled artisan.

According to a further embodiment, the isolated nucleic acid of the invention further comprises a nucleic acid specifying one or more regulatory sequences operably linked thereto.

In a further embodiment, the present invention includes a vector comprising a nucleic acid. This vector is preferably an expression vector which contains a nucleic acid according to the invention and one or more regulatory nucleic acid sequences.

Numerous vectors are known to be appropriate for the transformation of bacterial cells, for example plasmids and bacteriophages, like the phage  $\lambda$ , are frequently used as vectors for bacterial hosts. Viral vectors can be used in mammalian and insect cells to express exogenous DNA fragments, e.g. SV 40 and polyoma virus.

The transformation of the host cell can be done alternatively directly using "naked DNA" without the use of a vector.

The antibody/antibody fragment according to the invention can be produced either in eukaryotic or prokaryotic cells. Examples for eukaryotic cells include mammalian, plant, insect and yeast cells. Appropriate prokaryotic cells include *Escherichia coli* and *Bacillus subtilis*.

Preferred mammalian host cells are CHO, COS, HeLa, 293T, HEH or BHK cells or adult or embryonic stem cells.

Alternatively, the antibody/antibody fragment according to the invention can be produced in transgenic plants (e.g. potatoes, tobacco) or in transgenic animals, for example in transgenic goats or sheep.

According to a preferred embodiment, the vector is a plasmid or viral such as an engineered retrovirus or adeno virus.

According to a third aspect, the invention provides a pharmaceutical composition comprising an active component, preferably an antibody, a nucleic acid or a vector as defined hereinabove.

The active components of the present invention are preferably used in such a pharmaceutical composition in doses mixed with a pharmaceutically acceptable carrier or carrier material, that the disease can be treated or at least alleviated. Such a composition can (in addition to the

active component and the carrier) include filling material, salts, buffer, stabilizers, solubilizers and other materials, which are known state of the art.

The term "pharmaceutically acceptable" is defined as non-toxic material, which does not interfere with effectiveness of the biological activity of the active component. The choice of the carrier is dependent on the application.

The pharmaceutical composition can contain additional components which enhance the activity of the active component or which supplement the treatment. Such additional components and/or factors can be part of the pharmaceutical composition to achieve a synergistic or additional effects or to minimize adverse or unwanted effects.

Thus, the pharmaceutical composition of the invention may further contain one or more pharmaceutically active ingredients in order to supplement the treatment.

Preferably, such pharmaceutically active ingredients are selected from antibiotic agents, antiinflammatory agents, and / or agents blocking further pattern recognition receptors. Those agents preferably are specific for CD14, LBP, MD-2, TLR3, TLR4, TLR5, TLR7, TLR8, and/or TLR9.

Techniques for the formulation or preparation and application/medication of compositions of the present invention are published in "Remington's Pharmaceutical Sciences", Mack Publishing Co., Easton, PA, latest edition. A therapeutically effective dose relates to the amount of a compound which is sufficient to improve the symptoms, for example a treatment, healing, prevention or improvement of such conditions. An appropriate application can include for example oral, dermal, rectal, transmucosal or intestinal application and parenteral application, including intramuscular, subcutaneous, intramedular injections as well as intrathecal, direct intraventricular, intravenous, intraperitoneal or intranasal injections. The intravenous injection is the preferred treatment of a patient.

A typical composition for an intravenous infusion can be produced such that it contains 250 ml sterile Ringer solution and for example 1000 mg of the antibody of the invention. See also

Remington's Pharmaceutical Science (15. edition, Mack Publishing Company, Easton, Ps., 1980).

The active component or mixture of it in the present case can be used for prophylactic and/or therapeutic treatments.

An amount which is adequate to reach the aforesaid effect is defined as "therapeutically effective dose". Amounts, which are effective for these applications, depend on the severity of the condition and the general condition of the patient. However, the dose range is usually between 1 and 100 mg antibody per kilogram body weight of the patient in need of the treatment. Systemic application of a specific antibody of the invention, i.e. T2.5 (see experimental part) upon lipopeptide challenge inhibited inflammatory mediator release such as TNF $\alpha$  and prevented lethal shock-like syndrome in mice. 20 mg/kg of T2.5 was sufficient to protect mice and administration of 40 mg/kg of T2.5 was protective even 3 h after start of otherwise lethal challenge with *Bacillus subtilis*. Therefore, for treating mammals a dose of between 10 to 60 mg and more preferably between 20 to 40 mg per kilogram body weight is appropriate. Single or multiple applications after a daily, weekly or monthly treatment regimen can be performed with application rate and samples chosen by the physician in charge. These applications largely depend from the question, whether a chronic or acute condition is to be prevented or treated. An individual dose is administered as a single dose to the mammal suffering from an acute infection, wherein the individual dose is administered repeatedly to a mammal suffering from a chronic infection and/or inflammation.

The invention is additionally directed to a hybridoma which produces a monoclonal antibody as defined herein.

The antibody, nucleic acid, vector or composition of the present invention preferably are used in the prevention and/or treatment of inflammatory processes or any other process induced by bacterial infection, trauma, or chronic inflammation. Furthermore, they can be used for the prevention and/or treatment of bacteraemia or sepsis. The chronic infection preferably is selected from rheumatoid or vascular arthritis or inflammatory bowel disease.



The present invention in particular is directed to a gene therapy approach for use in the treatment of chronic diseases. The approach basically follows the already known protocols for gene therapy and comprises in particular the step of cloning a sequence comprising the variable domains of the antibody of the invention as specified above into an expression vector and introducing said expression vector into a host, for example a human patient in order to cause an overexpression of said antibody/antibody fragment in said patient.

According to a fourth aspect, the present invention is further directed to a screening method for identifying an antagonist capable of inhibiting or blocking TLR2, comprising the steps of:

- (a) generating or providing mammalian TLR2,
- (b) contacting said TLR2 with a candidate compound,
- (c) detecting the inhibition or blocking of said compound by a suitable detection method,
- (d) selecting a compound that has been tested positive in step (c),
- (e) optionally repeating steps (a) – (d) with a suitably modified form of the compound of step (d).

The present invention is in additionally directed to the following embodiments:

1. An antagonist, which specifically inhibits or blocks the mammalian, preferably human, Toll-like receptor 2 (TLR2).
2. The antagonist of emb. 1, which is an antibody, small molecule or an aptamer.
3. The antibody of emb. 2, which is a polyclonal antibody, a monoclonal antibody, a humanized antibody, a chimeric antibody, or a synthetic antibody.
4. The antibody of emb. 3, which is directed against the extracellular domain of TLR2.
5. The use of an antagonist of one or more of emb. 1-4 in the prevention and/or treatment of acute and/or chronic inflammatory processes induced by bacterial infection.

6. A screening method for identifying an antagonist capable of inhibiting or blocking TLR2, comprising the steps of:
- (f) generating or providing mammalian TLR2,
  - (g) contacting said TLR2 with a candidate antagonist,
  - (h) detecting the inhibition or blocking of said candidate antagonist by a suitable detection method,
  - (i) selecting a candidate antagonist that has been tested positive in step (c),
  - (j) optionally repeating steps (a) – (d) with a suitably modified form of the candidate antagonist of step (d).

The present invention will be further described with reference to the following figures and examples; however, it is to be understood that the present invention is not limited to such figures and examples.

**Fig. 1** Application of mAb T2.5 for specific detection of TLR2. Results of flow-cytometry of HEK293 cells stably overexpressing Flag-tagged mTLR2 (*a*) or human TLR2 (*b*), as well as primary *TLR2*<sup>-/-</sup> (*c*) and wild-type murine macrophages (*d*) by staining with mAb T2.5 (bold line, unfilled area). Negative controls represent cells not stained with a primary, but incubated with a mouse IgG specific secondary antibody (filled areas). For positive controls, Flag- (*a* and *b*) and mTLR2 (*c* and *d*) specific polyclonal antisera were used (normal line, unfilled area). For immunoprecipitation with T2.5, lysates of HEK293 cells overexpressing murine or human TLR2, as well as of murine RAW264.7 macrophages were applied as indicated (*e*). TLR2 precipitates were visualized by application of Flag (HEK293) or mTLR2 (RAW264.7) specific polyclonal antisera. Flag specific (αFlag) and protein G beads in the absence of antibodies (pG), as well as vector transfected HEK293 cells were used as controls. The size of TLR2 was 97 kDa.

**Fig. 2** Subcellular localization of TLR2 *in vitro*. MAb T2.5 was used for cytochemical detection of overexpressed murine and human TLR2 (*a*), as well as endogenous murine (*TLR2*<sup>+/+</sup>, wild-type) or human TLR2 in primary macrophages (*b*). Vector transfected

HEK293 cells, as well as *TLR2*<sup>-/-</sup> primary macrophages were analyzed as staining controls. Concanavalin A was used for staining of cytoplasmic membranes. The bars in the lower right corners of each field represent a distance of 20  $\mu$ m (*a*) or 10  $\mu$ m (*b*) on the slides analyzed.

**Fig. 3** Inhibitory effect of mAb T2.5 on cell activation *in vitro*. NF- $\kappa$ B dependent luciferase activities in HEK293 cells overexpressing either murine (*a*) or human TLR2 (*b*), as well as TNF $\alpha$  concentrations in supernatants of RAW264.7 (*c*) or primary murine macrophages (*d*) challenged with inflammatory stimulants are shown (ND, not detectable). Cells were incubated either with T2.5 or conT2 only (empty bars), or additionally challenged with IL-1 $\beta$  (*a*, *b*, horizontally hatched bars), ultra pure LPS (*c*, *d*, bold upward hatched bars), P<sub>3</sub>CSK<sub>4</sub> (filled bars), or h. i. *B. subtilis* (downward hatched bars, *a* to *d*). MAb and challenge (P<sub>3</sub>CSK<sub>4</sub>, LPS) dependent NF- $\kappa$ B / p65 nuclear translocation in human macrophages (*e*) was analyzed by cytochemical staining (Unstim., unstimulated). NF- $\kappa$ B dependent electro mobility shift assay (EMSA) and phosphorylation of MAP kinases Erk1/2 (pErk1/2) and p38 (pP38), as well as Akt (pAkt) were analyzed by applying nuclear or total extracts, respectively, from RAW624.7 macrophages (*f*, *g*). Cells were preincubated with the amounts of mAb T2.5 or conT2 indicated ( $\mu$ g/ml) and subsequently challenged with P<sub>3</sub>CSK<sub>4</sub> or LPS for 90 min (*f*, arrows indicate specific NF- $\kappa$ B-DNA complexes) or 30 min (*g*, P38, specific immunoblot as positive control). Untreated cells (control) were analyzed as controls.

**Fig. 4** TLR2 expression *in vivo*. Flow-cytometry of splenocytes and peritoneal washout cells from wild-type and *TLR2*<sup>-/-</sup> mice *ex vivo* immediately upon isolation (*n* = 5, cells pooled for each sample). CD11b<sup>+</sup> splenocytes from mice challenged with LPS for 24 h were analyzed for surface and intracellular TLR2 expression (*a*) by staining with T2.5 (bold line, *TLR2*<sup>+/+</sup>; filled area, *TLR2*<sup>-/-</sup>). For analysis of TLR2 regulation upon infection (*b*, *c*), mice were either left uninfected (-) or infected with Gram-positive *B. subtilis* and sacrificed after 24 h (+). Upon staining of CD11b, cells were stained with T2.5 (TLR2) either without (*b*) or upon permeabilization (*c*). Numbers in quadrants represent the proportion of single or double stained cells, respectively, as compared to the total number of viable cells analyzed (%).

**Fig. 5** Inhibitory effect of mAb T2.5 on host activation by microbial challenge *in vivo*. Mice were pretreated i. p. with 1 mg mAb T2.5 (solid bars) or left untreated (empty bars). Mice

were challenged after 1 h with P<sub>3</sub>CSK<sub>4</sub> and D-galactosamine (i. p.), as well as sacrificed 2 h or 4 h later ( $n = 4$  for each group at each time point). Serum concentrations of TNF $\alpha$  (a), GRO $\alpha$ /KC (human IL-8 homologue) (b), IL-6 (c), and IL-12p40 (d) were analyzed by ELISA (\* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.001$ , student's  $t$ -test for unconnected samples).

**Fig. 6** Effects of mAb T2.5 administration on viability upon TLR2-specific systemic challenging. IFN $\gamma$  and D-galactosamine sensitized mice received either no mAb, 1 mg of mAb T2.5, or 1 mg of conT.2 i. p. 30 min prior to microbial challenge with bacterial lipopeptide analogue P<sub>3</sub>CSK<sub>4</sub> (a, open circles, no mAb,  $n = 4$ ; open triangles, mAb conT2,  $n = 3$ ; filled squares, mAb T2.5,  $n = 4$ ). Mice challenged with a high dose of h. i. *B. subtilis* were left untreated or treated 1 h later with dosages of mAb T2.5 indicated (b, filled diamonds, 1 mg,  $n = 3$ ; open squares, 0.5 mg,  $n = 3$ ; open triangles, 0.25 mg,  $n = 4$ ; x's, 0.13 mg,  $n = 4$ ; open circles, no mAb T2.5,  $n = 4$ ) or with 1 mg of mAbs as indicated at different time points (c, d). Administration of TLR2-specific mAb prior (-), as well as after (+) bacterial challenge (c, filled inverted triangles, no mAb,  $n = 8$ ; open circles, mAb conT2, -1 h,  $n = 3$ ; filled diamonds, mAb T2.5, -1 h,  $n = 4$ ; open squares, mAb T2.5, +1 h,  $n = 3$ ; x's, mAb T2.5, +2 h,  $n = 3$ ; open diamonds, mAb T2.5, +3 h,  $n = 4$ ; open triangles, mAb T2.5, +4 h,  $n = 3$ ). Administration of TLR2-specific mAb T2.5 prior (-) to bacterial challenge (d,  $n = 3$  for experimental groups: open triangles, no mAb; filled squares, mAb T2.5, -3 h; open diamonds, mAb T2.5, -4 h; open circles, mAb T2.5, -5 h; filled inverted triangles, mAb T2.5, -6 h).

#### Fig. 7 FACS

- |    |                    |   |
|----|--------------------|---|
| 1, | Blocking           | 5% NGS and 1% Fcblock                               |
| 2, | Primary antibody   | Both TL2.1 and T2.5, 5 $\mu$ g/ml                   |
| 3, | Secondary antibody | FITC_Goat $\propto$ mouse IgG (Fab), 3.5 $\mu$ g/ml |

Conclusion: T2.5 specifically recognizes both murine and human TLR2 while TL2.1 interacts with human TLR2 and an unspecific antigen from murine cells in FACS analysis.

#### Fig. 8 and 9 Immunocytochemistry

- 1, Seed  $1 \times 10^5$  Cells/cover glass/ml/well on 24-well plate, culture overnight.

- 2, Fix cells with Methanol at  $-20$  degree for 8 minutes.
- 3, Wash with PBS for 3 times (dip into 3 beakers containing PBS).
- 4, Block with 2% NGS (normal goat serum in PBS) at 37 degree for 20 minutes in a humid chamber (20-30 $\mu$ l for each slide).
- 5, Incubate with T2.5/TL2.1 (5 $\mu$ g/ml) in 2% NGS at 37 degree for 60 minutes in a humid chamber (20-30 $\mu$ l for each slide).
- 6, Wash with PBS for 3 times (dip into 3 beakers containing PBS).
- 7, Incubate with secondary antibody (AlexaFluro546 conjugated goat anti mouse IgG, cat. A-11030, 8 $\mu$ g/ml, Molecular probes, Leiden, Netherlands) in 2% NGS at 37 degree for 60 minutes in a humid chamber (20-30 $\mu$ l for each slide).  
Option: Incubate with AlexaFluro488 conjugated Concanavalin A for cell surface staining (AlexaFluro488 conjugated Concanavalin A, cat. C-11252, 25 $\mu$ g/ml, Molecular probes, Leiden, Netherlands) in 2% NGS at 37 degree for 60 minutes in a humid chamber together with secondary antibody (20-30 $\mu$ l for each slide).
- 8, Wash with PBS for 3 times (dip into 3 beakers containing PBS).
- 9, Dry at RT for 20 minutes in dark, mount in mounting reagent, observe with confocol microscope (LSM510, carl Zeiss, Oberkochen, Germany).

**Conclusion** T2.5 specifically recognizes both murine and human TLR2 while TL2.1 interacts with human TLR2 and an unspecific antigen from murine cells in IHC analysis.

**Fig. 10 to 12** Dose dependent inhibition of cell activation by T2.5 but not by TL2.1 in both THP1 and RAW cells.

In order to analyze potential TLR2-inhibitory difference of TL2.1 and T2.5, human THP1 and murine RAW264.7 cells were used. Doses of both TL2.1 and T2.5 ranging from 5  $\mu$ g/ml to 40  $\mu$ g/ml as indicated in the figure were applied 30 min prior to challenge with P<sub>3</sub>CSK<sub>4</sub> at indicated concentrations. TNF $\alpha$  and IL-6 concentrations in supernatants of THP1 and RAW264.7 cells were analyzed 24 h after start of challenge.

**Fig. 13** Dose dependent binding of T2.5 to mTLR2ECD versus failed binding of TL2.1 to the same antigen in ELISA.

ELISA plates were coated with anti human IgG Fc antibody, human IgGFc fused mouse TLR2ECD protein was then immobilized after BSA blocking and washing followed by

detection with T2.5 and TL2.1 at various concentrations as indicated. Specific signals were visualized via application of HRP conjugated anti mouse IgG after primary antibody incubation followed by reaction with HRP substrates.

**Fig. 14** TL2.1 lacks binding capacity to overexpressed murine and human TLR2 in ELISA while T2.5 binds both in the same assay.

ELISA plates were coated with anti Flag antibody, Flag fused mouse and human TLR2 and control proteins were then immobilized after BSA blocking and washing followed by detection with T2.5 and TL2.1 at 1 µg/ml as indicated. Specific signals were visualized via application of HRP conjugated anti mouse IgG after primary antibody incubation followed by reaction with HRP substrates.

**Fig. 15** Lack of interaction between TL2.1 and murine TLR2 in Immunoprecipitation and subsequent immunoblot analysis.

Lysates of  $1 \times 10^6$  murine RAW264.7 macrophages or HEK293 cells, 1 µg of each antibody as indicated, and 20 µl of protein G beads (Santa Cruz, California, USA) were mixed for o. n. precipitation. Immune complexes were analyzed by immunoblot analysis with either Flag- or murine TLR2-specific antiserum.

**Fig. 16:** Antibodies (Abs) for immunoprecipitation (IP), as indicated, hT2.5 stands for linearized T2.5 heavy chain and light chain fused with C terminal human IgG1 Fc. Antigens (Ags) for IP: for lanes 1, 3, 5, 7, 9, lysates from HEK 293 cells overexpressing Flag-tagged mouse TLR2, for lanes 2, 4, 6, 8, 10, lysates from HEK293 cells without any transfection. A Western Blot (WB) was done with anti-Flag polyclonal sera. Bands at 97 kD resolved on 10 % SDS-PAGE gel indicate the position of Flag tagged murine TLR2 upon overexpression in HEK 293 cells precipitated with Abs.

**Fig. 16 A:**

A single chain and partially humanized TLR2-specific (hT2.5) antibody was generated by subcloning of a mammalian expression construct in the vector pcDNA3.1 within which expression is driven by the CMV promoter. From the 5' end of the MCS a sequence encoding a portion of the variable heavy chain (base

residue 1 to 450, see SEQ ID NO: 1), was followed successively by a linker sequence encoding 15 amino acids (Gly and Ser), a sequence encoding a portion of the variable light chain (base residue 61 to 396, see SEQ ID NO: 2), as well as the cds of the human Fc $\gamma$  (gamma) chain forming the 3' terminus (C terminus of the expressed hT2.5 protein) of the construct.

**Fig. 17:**

Molecular analysis of mAb T2.5 effects on TLR2ECD - P<sub>3</sub>CSK<sub>4</sub> interaction. Binding of recombinant TLR2ECD - Fc fusion protein (T2EC, positive controls) to immobilized P<sub>3</sub>CSK<sub>4</sub> upon pre-incubation with T2.5 (T2EC + T2.5) at different molar excesses (**a**, x 1, x 3.3, x 10) or with an isotype matched control mAb (T2EC + con) at 10 fold molar excess only (**b**, x 10). Binding was continuously monitored in a SPR biosensor device and amounts of antibodies used to gain high molecular excess over T2EC (co-incubation) were applied alone as negative controls (**a**, T2.5; **b**, con). Response units (RU) at 300 s are a measure for P<sub>3</sub>CSK<sub>4</sub>-binding capacities of T2EC and T2EC + mAb. For analysis of approximate localization of T2.5 epitope within the TLR2ECD, a mutant human TLR2 construct lacking the N-terminal third of the LRR-rich ECD domain (hTLR2-mutH) was used for NF- $\kappa$ B dependent luciferase assay upon transient transfection, preincubation with mAb (T2.5, conT2), and P<sub>3</sub>CSK<sub>4</sub> challenge (**c**, solid bars). Absence of mAb treatment (no mAb) and/or P<sub>3</sub>CSK<sub>4</sub> challenge (empty bars), as well as empty vector (Vector) represent respective controls (**c**).

**Fig. 18:**

Inhibitory effect of recombinant single chain and partially humanized hT2.5 antibody on lipopeptide induced cell activation. HT2.5 was overexpressed in HEK293 cells and supernatant collected (see. Fig. 16). HEK293 cells were either transfected with reporter plasmids only (open bars) or cotransfected with human TLR2 (black bars). Three groups of samples were established for both TLR2<sup>-</sup> and TLR2<sup>+</sup> cells as indicated. To samples of the first group, regular supernatant of normal HEK293 cells was administered. In the second group, cells were pretreated with culture supernatants of HEK293 cells overexpressing hT2.5. As positive control, application of 50  $\mu$ g/ml of T2.5 to supernatants in group three was performed. Specifically, cells of the groups one and two were pretreated by four times removal of supernatant and subsequent addition of conditioned supernatant for 10 min each

time while group three samples were treated by a single application of T2.5 to the original supernatants. One set of all samples was left untreated while a second set of samples was treated by application of lipopeptide analogue (P<sub>3</sub>CSK<sub>4</sub>) at a concentration of 100 ng/ml 40 minutes after antibody (T2.5 or hT2.5) incubation. NF- $\kappa$ B dependent reporter gene activation was analyzed 6 h upon start of lipopeptide challenge.

Result: The inhibitory effect of recombinant hT2.5 was similar to that of native T2.5 on TLR2-dependent cell activation.

Abbreviations used herein: TLR, Toll-like receptor; mAb, monoclonal antibody; P<sub>3</sub>CSK<sub>4</sub>, tripalmitoyl-cysteinyl-seryl-(lysyl)<sub>3</sub>-lysine; h. i. *B. subtilis*, heat inactivated *Bacillus subtilis*; sPGN, soluble peptidoglycan; LTA, lipoteichoic acid

## Examples

### Application of murine mAb T2.5 for expression analysis *in vitro*

The inventors selected an IgG1 $\kappa$  anti TLR2 mAb named T2.5 which specifically recognized TLR2. HEK293 cells stably expressing murine or human TLR2 were stained specifically on their surface by T2.5 (Fig. 1a and b). Furthermore, T2.5 did not bind to primary murine TLR2<sup>-/-</sup>, but bound to wild-type macrophages cultured *in vitro* (Fig. 1c and d). T2.5 immunoprecipitated native murine and human TLR2 from lysates of HEK293 cells overexpressing each of the two TLR2 orthologs (Fig. 1e). Most importantly, T2.5 precipitated endogenous TLR2 from lysates of RAW264.7 macrophages (Fig. 1e). Next, the inventors analyzed T2.5 for its capacity to specifically detect TLR2 on the subcellular level. Overexpressed murine and human TLR2 (Fig. 2a), as well as endogenous TLR2 were detectable in primary murine and CD14<sup>+</sup> leukocyte derived human macrophages (Fig. 2b).

### Inhibitory effects of T2.5 on TLR2 specific cell activation

T2.5 inhibited murine and human TLR2 mediated cell activation by TLR2 specific stimuli P<sub>3</sub>CSK<sub>4</sub> or *B. subtilis* applied to HEK293 cells overexpressing TLR2, murine RAW264.7, and



primary macrophages as measured by NF- $\kappa$ B dependent reporter gene assay and IL-8 specific enzyme linked immuno sorbent assay (ELISA), as well as TNF $\alpha$  and IL-6 specific ELISA, respectively (Fig. 3a to d and data not shown). A second newly generated IgG1 $\kappa$  anti TLR2 mAb, conT2, was used as a control. This mAb binds native murine (m) TLR2 in a manner comparable to T2.5 but not human TLR2 (data not shown) and failed to inhibit TLR2 dependent cell activation (Fig. 3). Also, no inhibition of Il-1 receptor or TLR4 signaling by T2.5 was detected, indicating that TLR2 independent signaling pathways in T2.5 treated cells remain intact (Fig. 3a to d). Moreover, TLR2 mediated nuclear translocation of NF- $\kappa$ B was specifically inhibited by T2.5 in human macrophages (Fig. 3e). NF- $\kappa$ B specific electro mobility shift assay (EMSA), as well as anti phospho p38, Erk1/2, and Akt immunoblot analysis revealed T2.5 but not conT2 dose dependent inhibition of P<sub>3</sub>CSK<sub>4</sub> induced NF- $\kappa$ B - DNA binding and cellular kinase phosphorylation (Fig. 3f and g).

#### **Flow-cytometry of intracellular and surface TLR2 expression ex vivo**

Since LPS induces TLR2 expression in primary macrophages *in vitro* (data not shown), the inventors first compared T2.5 specific staining of CD11b<sup>+</sup> splenocytes from LPS challenged wild-type and *TLR2*<sup>-/-</sup> mice by flow-cytometry. Weak surface staining and pronounced intracellular staining were seen (Fig. 4a). In subsequent experiments, peritoneal cells and splenocytes from mice infected with the Gram-positive bacterium *B. subtilis* were analyzed. While surface expression of TLR2 in primary murine macrophages was relatively strong upon *in vitro* culture (Fig. 1d), surface expression was weak or not detectable in unchallenged CD11b<sup>+</sup>, CD11c<sup>+</sup>, CD19<sup>+</sup>, and GR1<sup>+</sup> subpopulations of splenocytes and peritoneal washout cells (Fig. 4a, b and data not shown). Upon microbial challenge, however, TLR2 surface expression strongly increased in CD11b<sup>+</sup> and GR1<sup>+</sup> cells (Fig. 4b and data not shown). Signals were specific as tested by analysis of *TLR2*<sup>-/-</sup> cells (Fig. 4). Again, intracellular staining of TLR2 revealed significant levels of intracellular TLR2 expression which increased to a higher degree than surface expression upon microbial challenge (Fig. 4a and b).

#### **Antibody mediated interference with TLR2 specific immune responses towards systemic challenge**

Next, the inventors determined cytokine and chemokine serum concentrations in mice, either pretreated, or not pretreated with T2.5, as well as challenged with P<sub>3</sub>CSK<sub>4</sub>. While cytokine

and chemokine concentrations were low in sera of untreated mice (see methods), serum levels of TNF $\alpha$ , IL-8, IL-6, and IL-12p40 were significantly lower in mice preinjected with T2.5 as compared to controls and measured after challenge (Fig. 5a to d).

Both, a high dose (microbial product only) and a low dose model (additional sensitization with D-galactosamine) have been established for bacterial product induced shock in mice<sup>26</sup>. In order to interfere in a strictly defined model of septic shock, the inventors applied the bacterial lipopeptide analogue and TLR2 agonist P<sub>3</sub>CSK<sub>4</sub> upon sensitization of mice with interferon gamma (IFN $\gamma$ ) and D-galactosamine<sup>27</sup>. Sensitization was used to mimic priming of a host defense towards further microbial challenges by an underlying infection. While mice that had received no mAb or conT2 30 min prior to injection succumbed to lethal shock within 24 h, mice treated with T2.5 survived (Fig. 6a). Intending to employ a complex challenge mimicking infection for a distinct shock model, the inventors took advantage of the finding that shock induction by viable or heat inactivated Gram-positive *B. subtilis* bacteria is TLR2 dependent not only in a low dose, but also in a high dose model (unpublished observation). Mice were pretreated with T2.5 or conT2 followed by challenge with a lethal dose of *B. subtilis* (protective protocol). In a separate group of mice, the inventors first administered *B. subtilis* and applied T2.5 up to 3 h later (therapeutic protocol). In the absence of T2.5 the high dose *B. subtilis* challenge was lethal for all mice tested (Fig. 6b). However, when given T2.5 either prior (1 h), or up to 2 h after microbial challenge, all *B. subtilis* challenged mice survived. Most notably, treatment with T2.5 even 3 h after potentially lethal injection saved 75% of mice challenged (Fig. 6b).

The results indicated herein show a therapeutically useful function of an antagonistic TLR2 mAb in TLR2 driven septic shock. The inventors found that application of TLR2 agonists was lethal in two experimental models of septic shock and therefore aimed at identification of antibodies blocking TLR2. Here the inventors show that the mAb T2.5 prevents P<sub>3</sub>CSK<sub>4</sub>, a synthetic analogue of bacterial lipopeptides, or Gram-positive bacteria (*B. subtilis*) induced shock in mice. T2.5 also blocks human TLR2 function, since subcellular NF- $\kappa$ B translocation upon TLR2 specific challenge of primary human macrophages was inhibited upon its application.

The lack of TLR functions negatively affects humans at least upon acute infections<sup>28,29</sup>. In a systemic model of polymicrobial sepsis encompassing standardized influx of the gut flora into the peritoneal cavity, however, mice benefit from the lack of TLR functions<sup>30</sup> indicating TLR dependent mediation of harmful effects in acute infection. Indeed, blockade or application of LPS binding proteins such as CD14, bactericidal/ permeability-increasing protein (BPI), or LPS binding protein (LBP) has been effective to inhibit LPS induced pathology<sup>31-34</sup> even after LPS application. Since this exemplified the prevalent role of a single cellular system for specific recognition of a microbial product<sup>35,36</sup>, the inventors attempted to intervene in cell activation by TLR2 specific microbial products. The finding that T2.5 recognized murine and human TLR2 on the cell surface of and within cells cultured *in vitro*, as well as exhibited antagonistic effects upon application to cells expressing these receptors provided a basis for our attempt. Furthermore, the antagonistic function was specific and dose dependent.

The inventors analyzed the potential of T2.5 to prevent TLR2 driven immunopathology. Application of T2.5 30 min or 1 h prior to application of lethal doses of TLR2 specific agonists P<sub>3</sub>CSK<sub>4</sub> to sensitized mice or of *B. subtilis* to normal mice, respectively, protected mice against their lethal effects (Fig. 6a and b), but not against the lethal effects of LPS (data not shown). In fact, *B. subtilis* induced shock was prevented upon application of T2.5 2 h, or even 3 h after shock induction (100% or 75% of survival, respectively). Their results indicate complement mediated depletion of TLR2<sup>+</sup> cells as an unlikely mechanism of T2.5 mediated shock prevention, since application of the mTLR2 specific isotype matched mAb conT2 *in vivo* did not result in protection. This implicates reversibility of mAb mediated TLR2 blockade which is potentially important for timely recovery of TLR2 dependent cellular responsiveness in later phases of sepsis at which diminished immune function is fatal<sup>9</sup>. The demonstration of beneficial effects of T2.5 in both, a sensitization dependent and a high dose TLR2 specific experimental model, validate a therapeutical application in the face of many animal models being restricted to one of the two respective dosages. This characteristic may improve transferability of the results from our animal study to treatment of sepsis in humans<sup>9</sup>.

Perhaps it is the surprisingly very low constitutive surface expression of TLR2 in host cells such as CD11b<sup>+</sup> (macrophage), GR1<sup>+</sup> (granulocyte), CD19<sup>+</sup> (B cell), CD11c<sup>+</sup> (dendritic cell) splenocytes, and peritoneal cells *in vivo* (Fig. 4 and data not shown), which explains the

efficacy of T2.5 mediated prevention of shock triggered via TLR2. This low surface expression is in contrast with relatively high surface expression in unchallenged primary murine (Fig. 1), as well as human myeloid cells upon *in vitro* culture<sup>37</sup> giving account for immediate TLR2 expression analysis *ex vivo*. However, comparison of TLR2-staining of non-permeabilized and permeabilized cells indicates that a major portion of TLR2 was localized in the intracellular compartment of murine CD11b<sup>+</sup> and GR1<sup>+</sup> cells, as well as human macrophages (Fig. 4c, unpublished observation and Fig. 2b). In fact, the inventors noted increased surface and intracellular TLR2 expression in specific cell populations 24 h after bacterial infection which was similar upon LPS challenge (Fig. 4 and data not shown). The time course of TLR2 regulation in distinct immune cells upon microbial contact needs to be investigated in more detail because it might determine the time frame in which TLR blockade based intervention strategies can be effective.

Antagonistic properties have recently been demonstrated *in vitro* also for two anti human TLR2 mAbs<sup>38,39</sup> possibly indicating distinct active complex formation of TLRs as compared to receptors for which agonistic antibodies have been identified. However, T2.5 interferes with the lipopeptide/TLR2 complex that induces cell activation, as well as recognizes a human TLR2 construct lacking the N-terminal third of the TLR2 LRR rich domain (Fig. 17c) the inventors found to be dispensable for cellular recognition of lipopeptides<sup>40</sup>. Thus, the epitope recognized by T2.5 must be located within the C-terminal portion of the TLR2ECD. The inventors expect that identification of the epitope will show its conservation between mice and humans. The potential of T2.5, for instance in combination with further inhibitors of inflammatory processes, to inhibit pathogenesis of clinically important infections awaits its evaluation. In conclusion, the data provided herein are the first to point out the potential of TLR2 specific antibody application as a therapeutic strategy to block TLR2 mediated cell activation in the course of acute infection through *in vivo* evidence.

## Methods

**Material.** Over night (o. n.) *B. subtilis* (DSMZ.1087) cultures containing approximately  $1 \times 10^{10}$  colony forming units (cfu)/ml (brain heart medium) were used immediately or heat inactivated (h. i.) at 56°C for 50 min. Synthetic P<sub>3</sub>CSK<sub>4</sub> was purchased from ECHAZ microcollections (Tuebingen, Germany), ultra pure LPS from *Salmonella minnesota Re595*

was from List Laboratory (Campbell, California), recombinant murine IFN $\gamma$  and IL-1 $\beta$  from Peptotech (London, England), and D-galactosamine from Sigma (Deisenhofen, Germany).

**Mice.** Matched groups of wild-type (*TLR2*<sup>+/+</sup>) B57BL/6 and *TLR2*<sup>-/-27</sup> mice kindly provided by Tularik (generated by Deltagen; South San Francisco, California; nine-fold crossed towards B57BL/6 background) were applied.

**Generation of TLR2ECD specific antibodies and ELISA.** A cDNA fragment encoding the N-terminal 587 amino acids of mTLR2<sup>41</sup> was amplified from a RAW264.7 cDNA library (advantage kit, BD Clontech, Heidelberg, Germany). The murine TLR2ECD was fused to a C-terminal thrombin cleavage site followed by a human IgGFc $\gamma$  moiety. The murine TLR2ECD protein was purified upon overexpression in HEK293 cells and thrombin digestion. A *TLR2*<sup>-/-</sup> mouse was immunized by intraperitoneal (i. p.) injection of 50  $\mu$ g of TLR2ECD and 10 nmol of a thioated DNA oligonucleotide (5'-TCCATGACGTTCTGA-3', Tib Molbiol, Berlin, Germany) for three times within eight weeks. Its splenocytes were fused with murine P3X cells and hybridomas were selected<sup>42</sup>. MAb specificities for TLR2ECD, as well as cyto- and chemokine concentrations in cell supernatants or murine sera (see below) were analyzed by ELISA (R&D systems, Minneapolis, Minnesota).

**Flow cytometry.** Stably transfected HEK293 cell clones, as well as uninduced peritoneal wash-out macrophages were cultured o. n. as described<sup>40</sup>. Flow cytometry was performed upon staining with either T2.5, or affinity purified polyclonal rabbit antisera specific for the murine TLR2ECD<sup>43</sup> or the Flag tag (Sigma), as well as respective secondary mAbs (BD Pharmingen, Heidelberg, Germany).

For establishment of mTLR2 expression analysis in primary cells, surface and intracellular T2.5 dependent staining of CD11b<sup>+</sup> splenocytes<sup>42</sup> from wild-type and *TLR2*<sup>-/-</sup> mice challenged with LPS (0.5 mg, i. p., 24 h) were compared by flow cytometry (CyAn, Dako Cytomation, Fort Collins, Colorado). Cells were stained with photoactivated ethidium monoazide (Molecular Probes, Amsterdam, Netherlands) immediately upon isolation, followed by TLR2 specific surface staining, or intracellular staining (cytofix/cytoperm, BD Pharmingen). In order to analyze TLR2 expression in non- or *B. subtilis* infected ( $5 \times 10^{10}$

cfu, i. p., 24 h) mice, peritoneal washout cells and splenocytes<sup>42</sup> from five wild-type or *TLR2*<sup>-/-</sup> mice were pooled, respectively. Fluorescence labeled cell surface marker antibodies (BD Pharmingen) and primary T2.5 stained with secondary anti mIgG1 were used as indicated.

**Immunoprecipitation and immunoblot analysis.** Lysates of Flag-TLR2 transfected HEK293 cells or macrophages, as well as 1 µg of antibody and protein G beads (Santa Cruz, California) were mixed for o. n. precipitation. Immune complexes or cell lysates were analyzed by immunoblot analysis as described<sup>40</sup>. Precipitations were controlled by application of Flag specific (mAb M2, Sigma) or protein G beads only. Flag (HEK293) or mTLR2<sup>43</sup> (RAW264.7) specific antisera were applied for immunoblot analyses. In contrast, total lysates of macrophages (see inhibition experiments) were analyzed for phosphorylation of kinases indicated.

**Cytochemical staining of TLR2 or NF-κB.** Transfected HEK293 cell clones, as well as primary murine or human macrophages, the last isolated as CD14<sup>+</sup> peripheral blood leukocytes and cultured in 20% of autologous serum<sup>44</sup>, were grown on slides. Cells were washed with PBS, permeabilized, and incubated with 50 µg/ml TLR2 specific mAb and/or anti NF-κB/p65 (polyclonal rabbit, Santa Cruz)<sup>40</sup>. Specific secondary αIgG antibodies were applied. Cell membranes were stained with labeled concanavalin A (Molecular Probes).

**Inhibition of TLR2 dependent cell activation *in vitro* and *in vivo*.** Transiently transfected HEK293 cells, murine RAW264.7, as well as primary macrophages were used<sup>40</sup>. 50 µg/ml of antibodies were applied 30 min prior to challenge with 100 ng/ml of LPS, IL-1β, P<sub>3</sub>CSK<sub>4</sub>, or 1 x 10<sup>6</sup> cfu/ml of h. i. *B. subtilis*. HEK293 cells were cotransfected with reporter<sup>45</sup>, human CD14, human or mTLR2, and MD2 (provided by Tularik, Drs. Golenbock and Heine, as well as Miyake, respectively) expression plasmids, and NF-κB dependent reporter gene activity was assayed after 6 h of stimulation<sup>40</sup>. TNFα concentrations in supernatants of RAW264.7 and primary murine macrophages, as well as NF-κB translocation in human macrophages<sup>44</sup> were analyzed 24 h and 90 min after challenge, respectively. For carrying out challenge and antibody dose dependent NF-κB dependent EMSA, as well as p38, Erk1/2, and Akt phosphorylation specific immunoblot analysis (Cell signaling, Frankfurt, Germany),

RAW264.7 macrophages were used.  $1 \times 10^6$  cells were pretreated with antibodies as described above at various concentrations and stimulated for 90 min (EMSA) or 30 min (kinase phosphorylation analysis)<sup>40</sup>. For analysis of TLR2 inhibition *in vivo*, mice were injected i. p. with 1 mg of T2.5 or left untreated. 1 h later, 100  $\mu$ g of P<sub>3</sub>CSK<sub>4</sub> and 20 mg of D-galactosamine were injected i. p. Serum concentrations of TNF $\alpha$ , IL-8, IL-6, and IL-12p40 in five unchallenged control mice were 0.05 ng/ml, 0.43 ng/ml, not detectable, and 0.44 ng/ml, respectively.

**Systemic shock induction.** In an experimental sensitization dependent model<sup>27</sup>, mice were injected intravenously with 1.25  $\mu$ g of murine IFN $\gamma$ . 20 min later, mice were injected i. p. with 1 mg of mAb as indicated. 50 min after IFN $\gamma$  injection, 100  $\mu$ g of synthetic P<sub>3</sub>CSK<sub>4</sub> and 20 mg of D-galactosamine were injected i. p. as well. The experimental high dose shock model encompassed a single i. p. injection of h. i. *B. subtilis* suspension (corresponding to  $5 \times 10^{10}$  cfu) with prior (1 h) or subsequent (1 h, 2 h, or 3 h) i. p. injection of 1 mg mAb as indicated.

### Material and method (for Fig. 17)

*Abrogation of TLR2ECD ligand-binding by T2.5 and analysis of T2.5 epitope localization.* To investigate whether T2.5 blocked binding of TLR2 to its synthetic agonist P<sub>3</sub>CSK<sub>4</sub> the inventors established a SPR biosensor based binding assay. P<sub>3</sub>CSK<sub>4</sub> was immobilized on a chip surface and binding of T2EC was tested under various conditions. PHCSK<sub>4</sub>, a non-active analogue of P<sub>3</sub>CSK<sub>4</sub>, was used as control and sensograms are displayed as subtracted binding curves. Binding of T2EC to P<sub>3</sub>CSK<sub>4</sub> was specific (Figure 17a). When T2EC was pre-incubated with T2.5, the antibody dose dependently inhibited T2EC - P<sub>3</sub>CSK<sub>4</sub> binding (Figure 17a). A molar ratio of 3.3 (T2.5 : T2ECD) was required to reduce binding to 50%. Pre-incubation of T2EC with T2.5 at a 10 fold molar excess abrogated T2EC - P<sub>3</sub>CSK<sub>4</sub> interaction (Figure 17a). In contrast, an isotype matched control antibody did not block binding of T2EC to P<sub>3</sub>CSK<sub>4</sub> even if applied at 10 fold molar excess (Figure 17b). If applied alone, both mAbs did not interact with the sensor-chip surface (Figure 17, a and b). The N-terminal third of the LRR-rich domain of human TLR2 is not involved in lipopeptide recognition (37) and T2.5 cross-reacts with human TLR2 (Figures 2b and 3e). Thus, we applied T2.5 to HEK293 cells

overexpressing a mutant construct of human TLR2 lacking the respective portion of the wild-type ECD (37). Specific abrogation of NF- $\kappa$ B dependent reporter gene activation upon P<sub>3</sub>CSK<sub>4</sub> challenge after administration of T2.5 strongly suggests localization of the epitope recognized by T2.5 within the C-terminal portion of the TLR2ECD (Figure 17c).

Direct interaction between TLR2 and P<sub>3</sub>CSK<sub>4</sub> was demonstrated and allowed comparison of TLR2 and TLR2-T2.5-complex affinities to this ligand. SPR analysis showed the direct and specific interaction between TLR2ECD and P<sub>3</sub>CSK<sub>4</sub>, as well as a specific and dose dependent inhibition of this interaction by T2.5 (Figure 17, a and b), indicating that binding of T2.5 masked the ligand-binding domain in TLR2. Accordingly, T2.5 antagonized not specifically P<sub>3</sub>CSK<sub>4</sub>, but also h. i. *B. subtilis*, PGN, LTA, and mycoplasmal macrophage activating protein induced TLR2-dependent cell activation (Figure 3 and data not shown). Blockage was specific and dose dependent (Figure 3). Taken together, these findings show that specific binding of ligands to a discrete site within the TLR2ECD is a prerequisite for TLR2 mediated signaling.



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